AVIAN DISEASES 54:848–856, 2010

Subgroup J Avian Leukosis Virus Neutralizing Antibody Escape Variants Contribute to Viral Persistence in Meat-Type Chickens

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Received 26 September 2009; accepted and published ahead of print 28 December 2009

SUMMARY. We have previously demonstrated a high incidence of chickens with persistent viremia even in the presence of neutralizing antibodies (V+A+) against the inoculated parental virus in commercial meat-type chickens inoculated at hatch with subgroup J avian leukosis virus (ALV J) field isolates. In this study, we used an ALV J molecular clone, ADOL pR5-4, to determine the role of neutralizing antibody (NAb) escape mutants in maintaining a high incidence of viral persistence, namely, V+A+ infection profile in commercial meat-type chickens. Chickens were housed as a flock in a pen or housed in isolation in solitary Horsfall-Bauer units for testing for NAb escape variants. The emergence of NAb escape variants was evaluated by sequential autologous virus neutralization (VN) (between virus and antibody from the same sampling period) and heterologous VN (between virus and antibody from preceding and succeeding sampling periods). Sequential virus isolates and corresponding antisera from 18 chickens were examined by VN matrix. In all chickens, autologous virus isolates were not neutralized by corresponding antisera. However, some of these resilient autologous virus isolates were neutralized by antibodies from subsequent sampling intervals. Nucleotide sequence analysis of consecutive isolates from three individually housed chickens with V+A+ infection profile revealed distinct changes within the envelope region, suggesting viral evolution to escape the host immune response. These results demonstrate that the emergence of antibody escape variants in commercial meat-type chickens contributes to ALV J persistence.

RESUMEN. Los virus variantes que escapan de los anticuerpos neutralizantes contra el virus de la leucosis aviar subgrupo J contribuyen a la persistencia viral en pollos de engorde.

Se ha demostrado con anterioridad, una alta incidencia de pollos con viremia persistente, incluso en presencia de anticuerpos neutralizantes (perfil V+A+) contra el virus paterno en pollos de engorde comerciales inoculados al día de edad con aislamientos de campo del virus de la leucosis aviar subgrupo J (ALV J). En este estudio, se utilizó un clon molecular del virus de la leucosis subgrupo J, denominado ADOL PR5-4, para determinar el papel de los virus mutantes que escapan de los anticuerpos neutralizantes, en el mantenimiento de una alta incidencia en la persistencia viral; es decir, en pollos de engorde comerciales que presentan el perfil V+A+. Los pollos fueron alojados como una parvada en un corral o alojados de manera individual en unidades de aislamiento tipo Horsfall-Bauer para la detección de variantes de escape a los anticuerpos neutralizantes. Se evaluó la aparición de los virus variantes de escape mediante virus-neutralización autóloga secuencial (entre el virus y anticuerpos del mismo periodo de muestreo) y por virus neutralización heteróloga (entre el virus y anticuerpos de muestreos anteriores y posteriores). Se examinó la serie de aislamientos virales y de anticuerpos correspondientes que fueron obtenidos de 18 pollos mediante una matriz de virus neutralización. En todos los pollos, los aislamientos virales autólogos no fueron neutralizados por los antisueros correspondientes. Sin embargo, algunos de estos aislamientos virales autólogos resistentes fueron neutralizados por anticuerpos recolectados en muestreos posteriores. El análisis de las secuencias de nucleótidos de los aislamientos consecutivos de tres pollos alojados individualmente que mostraban un perfil de infección V+A+ reveló distintos cambios en la región de la envoltura, lo que sugiere el desarrollo de una evolución viral para escapar de la respuesta inmunitaria del huésped. Estos resultados demuestran que la aparición de variantes de escape de anticuerpos en los pollos de engorde comerciales contribuye a la persistencia del virus de la leucosis aviar subgrupo J.

Key words: subgroup J avian leukosis virus, antibody escape mutants, meat-type chickens, persistent viremia, neutralizing antibody, infection profile

Abbreviations: AA = amino acid; ADOL = Avian Disease and Oncology Laboratory; ALV = avian leukosis virus; ALV J = subgroup J avian leukosis virus; CEF = chicken embryo fibroblast; C/E = cells resistant to subgroup E avian leukosis virus; CS = calf serum; EIAV = equine infectious anemia virus; ELISA = enzyme-linked immunosorbent assay; FMDV = foot and mouth disease virus; gsa = group-specific antigen; HB = Horsfall-Bauer; HIV = human immunodeficiency virus; KEDTA = potassium ethylenediaminetetraacetic acid; LCMV = lymphocytic choriomeningitis virus; LM = Leibowitz L-15-McCoy 5A tissue culture medium; LTR = long terminal repeat; NAb = neutralizing antibody; NS = nonsynonymous; PH = posthatch; PI = postinoculation; S = synonymous; SU = surface; $TCID_{50}$ = 50% tissue culture infectious dose; TM = transmembrane; VI = virus isolation; VN = virus microneutralization

Subgroup J avian leukosis virus (ALV J) causes a variety of neoplastic and nonneoplastic conditions primarily in meat-type chickens and is responsible for severe losses to the poultry industry. ALV J is largely controlled by identification and eradication of chickens positive for antibody or viremia depending on the eradication protocols followed by the breeder. As with other retroviruses, ALV J is capable of persisting at very low levels in the

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The several *env* gene sequences of subgroup J ALV ADOL pR5-4 and its antibody escape variants described in this manuscript have been deposited in GenBank and assigned the following accession numbers: GU222396–GU222420.

host, and the routinely used diagnostic methods may not always detect the virus (12). Hence, the real prevalence of ALV J may be actually higher than what has been reported.

The ALV J infection profile and transmission pattern in meat-type chickens is comparable to that of other exogenous avian leukosis virus (ALV) infections (14,15,17,25). In general, chickens infected *in ovo* with ALV result in tolerant viremia with no apparent immune response against the inoculated virus (V+A−), and chickens infected after hatch may clear viremia by producing an efficient neutralizing antibody (NAb) response against the inoculated virus (V−A+). However, meat-type chickens infected with ALV J during the first 2 wk after hatch generally have high levels of viral persistence in the absence (V+A−) or presence of NAb (V+A+) (3,13,26). The continued viral persistence that is often observed in chickens that develop NAb against the inoculated virus (V+A+) might indicate the presence of viral strains different from the inoculated viral strain or NAb escape variants.

The role of NAb escape variants in viral persistence has been demonstrated in other viruses, especially equine infectious anemia virus (EIAV) (6), visna virus (10), human immunodeficiency virus (HIV) (24), lymphocytic choriomeningitis virus (LCMV) (1), and foot and mouth disease virus (FMDV) (8). The significance of NAb escape variants in ALV J–induced persistence has not been evaluated.

We have previously demonstrated a high incidence of chickens with persistent viremia even in the presence of neutralizing antibodies (NAb) against the inoculated parental virus (V+A+) in commercial meat-type chickens inoculated at hatch with ALV J field isolates. However, the factors responsible for this high V+A+ incidence in meat-type chickens in not really clear.

The objectives of this work were to confirm the high incidence of V+A+ infection profile in meat-type chickens infected at hatch and at 1 wk posthatch (PH) with an ALV J molecular clone ADOL pR5-4 as demonstrated in previous experiments with ALV J field isolates; to demonstrate NAb escape variants in V+A+ chickens infected with ALV J field isolates as well as with ADOL pR5-4 by evaluating sequential autologous and heterologous NAb responses; and to sequence the consecutive viral isolates from individual chickens infected with ADOL pR5-4 and reared in isolation.

MATERIALS AND METHODS

Chickens. Commercial meat-type chickens and ADOL line 0 chickens (2) were used. Chicken embryos from ALV-free tested commercial broiler breeder hens were hatched in house. Blood and/or meconium from day old chicks were tested by virus isolation to confirm the ALV-free status. The experimental ADOL line 0 chickens are specific-pathogen-free chickens. In Experiment 1, chickens were housed in floor pens maintained as isolation units under biosecurity level-2 containment for 32 wk. Line 0 chickens were provided feed and water *ad libitum*, but for commercial meat-type chickens feed was restricted to limit excess body weight gain as recommended by the breeder. In Experiment 2, commercial meat-type chickens were housed in Horsfall-Bauer (HB) units and reared in isolation for 20 wk.

Viruses. Three ALV J field isolates (ADOL Hc1, ADOL 6803, and ADOL 4817) were selected based on geographic origin and nucleotide sequence differences (3). All the ALV J strains were isolated from separate meat-type chicken farms across the United States. In addition, a molecularly cloned ALV J, ADOL pR5-4 derived from a field ALV J strain ADOL R5-4 (7), was used in this study. The ADOL pR5-4 viral clone was modified to contain a splice acceptor site in place of the redundant transmembrane domain as well as a unique restriction site for cloning of foreign genes (7). ADOL pR5-4 was demonstrated to have similar biological characteristics to other ALV J strains (7). All viruses were propagated in ADOL line 0 known to be resistant to subgroup E ALV (C/E) secondary chicken embryo fibroblasts (CEFs) that support propagation of all exogenous ALVs. The virus titers were determined by limiting dilution in tissue culture. The titers varied from $10^{5.5}$ to $10^{6.5}$ 50% tissue culture infectious dose ($TCID_{50}$) per milliliter.

Experimental design and sample selection. Sequential samples from five chickens with V+A+ infection profile from our previous experiments (3,13,26) were used for testing for antibody escape variants. In Experiment 1, we inoculated 50 commercial meat-type chickens and 100 ADOL line 0 chickens intra-abdominally at hatch with 1000 $TCID_{50}$ ADOL pR5-4. In addition, 22 meat-type chickens were housed as sentinels. The chickens were bled at 1, 8, 16, 24, 28, and 32 wk postinoculation (PI). At sampling, 3–5 ml of blood was collected in syringes coated with potassium ethylenediaminetetraacetic acid (KEDTA) and spun at 640 × *g* for 30 min to separate the plasma. All samples were placed on melting ice immediately after collection and assayed fresh or stored at −70 C until assayed.

In Experiment 2, we inoculated 10 commercial meat-type chickens *in ovo* by yolk sac route at 8 days of embryonic age with 100 $TCID_{50}$ ADOL pR5-4. Another 30 chickens were inoculated intra-abdominally at 1 wk of age with 100 $TCID_{50}$ ADOL pR5-4. Chickens inoculated *in ovo* and at 1 wk of age were housed in separate Horsfall-Bauer isolation units with four noninfected age-matched chickens (cage mates) for the first 2 wk to support social interaction as recommended by the ADOL Animal Care and Use Committee. After 2 wk, the additional noninfected age-matched chickens were removed from the Horsfall-Bauer isolation units and the infected chickens were housed in isolation for the remainder of the experiment. All chickens inoculated *in ovo* and at 1 wk of age were bled at 0, 2, 4, 8, 12, 16, and 20 wk PI. The blood samples were processed as described in Experiment 1.

To evaluate NAb escape variants, five chickens from a previous experiment (13) that were inoculated with ALV J field isolates and raised as flocks in floor pens (ADOL Hc1, ADOL 4817, and ADOL 6803), ten chickens inoculated with ADOL pR5-4 and raised as a flock in a floor pen (from Experiment 1), and three chickens inoculated with ADOL pR5-4 and raised in isolation in individual HB units (from Experiment 2) with V+A+ infection profile were selected for testing by VN matrix. All the 18 V+A+ chickens that were selected had persistent V+A+ infection profile on the last four to six sampling intervals (Table 1). In addition, samples from three chickens (10022, 10075, 10089) in Experiment 2 that were selected for VN matrix were also selected for nucleotide sequence analysis.

Virus isolation (VI). Plasma samples collected during each sampling period were tested for viremia by VI. Samples were tested according to the procedures described earlier (4). Briefly, about 100 µl of undiluted plasma was added to 1.8×10^5 CEFs suspended in 4% calf serum (CS) Leibowitz L-15-McCoy 5A tissue culture medium [1:1] (LM) containing penicillin, streptomycin, amphotericin B, and 0.004 IU heparin in 24-well tissue culture plates. On the following day, the 4% CS LM media was replaced with 1% CS LM media. The plates were incubated in 4% CO_2 at 37 C for 7–9 days before the cells were completely lysed with 50 µl of 0.5% tween 80 (Sigma Chemical Co., St. Louis, MO) and two alternate cycles of freezing at −70 C and thawing at 37 C. About 100 µl of the cell lysate was used to test for p27 group-specific antigen (gsa) by enzyme-linked immunosorbent assay (ELISA) (20). The p27 gsa ELISA was carried out using rabbit anti-p27 polyclonal antibody coated Immulon® plates (Dynatech, Chantilly, VA), rabbit anti-p27 antibody conjugated to horseradish peroxidase (SPAFAS, Storrs, CT), and 3, 3′, 5, 5′-tetramethyl benzidine substrate (BD Biosciences Pharmingen, San Diego, CA). The plate was read at an absorbance of 630 nm using a MRX microplate reader (Dynex, Chantilly, VA).

Virus microneutralization (VN). Plasma samples were tested for NAb against ALV J viral stocks that were used to infect the experimental chickens. VN assays were performed as described earlier (4). In précis, the plasma samples were diluted 1:5 in serum-free LM media and incubated at 56 C for 30 min to denature the complement factors. About 500–1000 ALV J viral particles in 50 µl of LM media are incubated with 50 µl of heat-denatured 1:5 diluted plasma in 96-well flat bottomed tissue culture plates for 45 min in 4% CO_2 at 37 C. After the incubation, about 1×10^5 cells in 150 µl of 4% CS LM media were pipetted into each of the 96 wells and incubated at 37 C and 4% CO_2 for 7–9 days. At the end of

Table 1. Heterologous and autologous virus neutralization (VN) profile of commercial meat-type chickens after ALV J infection.

Chicken[A]	Samples[A]	Virus	V+A+ incidence[B]	Autologous VN[C]
1	Retrospective	ADOL Hc1	4/4	0/4
2	Retrospective	ADOL Hc1	4/4	2/4
3	Retrospective	ADOL 4817	4/4	1/4
4	Retrospective	ADOL 4817	4/4	0/4
5	Retrospective	ADOL 6803	4/4	0/4
6	Expt. 1	ADOL pR5-4	6/6	2/6
7	Expt. 1	ADOL pR5-4	5/5	2/5
8	Expt. 1	ADOL pR5-4	5/5	2/5
9	Expt. 1	ADOL pR5-4	6/6	4/6
10	Expt. 1	ADOL pR5-4	5/5	0/5
11	Expt. 1	ADOL pR5-4	5/5	3/5
12	Expt. 1	ADOL pR5-4	6/6	1/6
13	Expt. 1	ADOL pR5-4	5/5	2/5
14	Expt. 1	ADOL pR5-4	6/6	2/6
15	Expt. 1	ADOL pR5-4	6/6	2/6
16	Expt. 2	ADOL pR5-4	4/4	0/5
17	Expt. 2	ADOL pR5-4	4/4	0/5
18	Expt. 2	ADOL pR5-4	4/4	0/5

[A]Commercial meat-type chickens (1–5) were infected with various ALV J field isolates (from a previous study Pandiri *et al.*, 13), and chickens (6–15) from Experiment 1 were infected with ALV J molecular clone ADOL pR5-4 at hatch and raised as flocks in a floor pens. Chickens (16–18) from Experiment 2 infected with ALV J molecular clone ADOL pR5-4 at 1 wk posthatch and raised in isolation in individual HB units.

[B]Heterologous VN between the sample antibody and the inoculated parental virus. Number of sampling intervals positive for heterologous neutralization/total number of sampling intervals for each chicken.

[C]Autologous VN between antibody and virus from the same sampling interval. Number of sampling intervals positive for autologous neutralization/total number of sampling intervals for each chicken.

incubation, the cell cultures were completely lysed with 20 µl of 0.5% tween 80 (Sigma Chemical Co.) and were subjected to two alternate cycles of freezing at −70 C and thawing at 37 C. The cell lysates were tested for p27 gsa ELISA as described earlier (20). Samples that had a chromogenic reading of 1 or negative on the p27 gsa ELISA readout were considered to be positive for NAb against ALV J, and samples with a chromogenic reading of ≥2 on the p27 gsa ELISA readout were considered to be negative for NAb against ALV J.

Design of VN assays. The V+A+ infection profile is based on serum NAb isolated at various sampling intervals against the inoculated parental virus. Sequential plasma samples from each chicken with consistent V+A+ infection profile were inoculated into line 0 C/E CEFs. The virus stocks were prepared and titrated by limiting dilution in tissue culture. The plasma samples were diluted 1:2, 1:5, 1:10, and 1:20 in serum-free LM media and heat-denatured at 56 C for 30 min. Each of these virus stocks and plasma samples (antibodies) were subjected to duplicate autologous VN (between virus and antibody from the same sampling) or heterologous VN (between virus and antibody from preceding and succeeding samplings). A VN matrix was constructed based on autologous and heterologous VN patterns using 1:5 diluted plasma samples. In addition, a cross VN matrix assay was also conducted using the sequential virus isolates from chicken 10055 and antibody samples from chicken 10075 and *vice versa*, from Experiment 2.

PCR amplification, DNA sequencing, and analysis. Proviral DNA was isolated from passage 3 virus grown on line 0 CEFs from serial bleeds from chickens 10075, 10089, and 10022. The entire envelope region and redundant, D, E, and long terminal repeat (LTR) regions were amplified by polymerase chain reaction using primers 6–2 (19). The oligonucleotide primers were synthesized by Operon Technologies (Alameda, CA). The ALV J–specific primers include the forward primer 6 (5′-CTT GCT GCC ATC GAG AGG TTA CT-3′), annealing to gp85, and the reverse primer 2 (5′-AGT TGT CAG GGA ATC GAC-3′), annealing to sequences within the LTR. All the PCRs were in 50-µl volumes and contained 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1 mg/ml nuclease free bovine serum albumin, 2.5 units *Pyrococcus furiosus* polymerase (Stratagene, La Jolla, CA), a 200 mM concentration of each deoxynucleoside triphosphate, 25 pmol of each primer, and 50–200 ng proviral DNA. Following an initial template melting step at 95 C for 3 min, the DNA was amplified during 30 cycles of 95 C for 1 min, 57 C for 1 min, and 72 C for 2 min. A final elongation step at 72 C for 10 min completed the PCR.

The PCR products were purified by gel electrophoresis and Geneclean® (QBIOgene, Solon, Ohio) procedure before cloning into the pCR® blunt cloning vector for sequencing of the envelope region. Plasmids were purified using the S.N.A.P.™ Miniprep Kit before sequencing using primers 2 and 6. Sequences of at least two to three independent clones from at least two different PCR reactions were sequenced using an ABI model 373A automatic DNA sequencer (Applied Biosystems, Foster City, CA), and contigs were constructed using Sequencer (Gene Codes Corp., Ann Arbor, MI). Nucleotide and protein sequences were aligned using the ClustalX method (5), as implemented in MegAlign (DNAStar Inc., Madison, WI). The phylogenetic relatedness of the isolates was calculated using bootstrap analysis with the maximum-parsimony method, as used in Phylogeny Inference Package (PHYLIP, version 3.6, 2004, distributed by J. Felsenstein, Department of Genetics, University of Washington, Seattle, WA). The molecular evolution of the variant viruses was examined by measuring the relative rates of amino acid (AA) change, as well as calculating the N-glycosylation sites within the *env* gene region. The N-glycosylation sites (Asn-Xaa-Ser/Thr) were calculated using the NetNGlyc 1.0 server from the University of Denmark website. The relative rates of AA change was measured by calculating the ratio of nonsynonymous (NS, AA changing) to synonymous (S, AA preserving) AA substitution patterns based on the methods described by Nei and Gojobori (11).

Statistics. Statistical analysis was performed by testing for significance of differences in percentages by chi square test using Statistica® (Statsoft, Tulsa, OK). Statistical significance was assumed at less than 0.05 level of probability. Sequences were aligned by using the ClustalX method (5). The Jones-Taylor-Thornton probability model of change between AAs along with PHYLIP, version 3.6 maximum likelihood method for AA sequences was used to generate the phylogenic dendrogram.

Table 2. Infection profile of commercial meat-type chickens infected with ALV J molecular clone ADOL pR5-4 at day of hatch (Experiment 1).

Lot	Chicken strain	Inoculation dose ($TCID_{50}$)	ALV J infection profile[A,B] V+A− (%)	V+A+ (%)	V−A+ (%)	Chickens
1	Meat-type	1000	12[a]	88[a]	0[a]	50
2	Meat-type	Contacts	0[b]	23[b]	77[b]	22
3	ADOL line 0	1000	3[b]	4[c]	93[c]	100

[A]Virus isolation (V); neutralizing antibody against the inoculated parental virus (A) V+A− = chickens that were consistently positive for viremia and did not develop any neutralizing antibody; V+A+ = chickens that remained viremic at the end of the study and were concurrently positive for viremia and neutralizing antibody response on at least one occasion; V−A+ = chickens that have successfully seroconverted with loss of viremia and developed neutralizing antibodies by the time the study was terminated.

[B]Alphabetic superscripts indicate statistically significant differences at $P < 0.05$ level.

Table 3. Infection profile of commercial meat-type chickens (30) following infection with a molecularly cloned ALV J strain, ADOL pR5-4, at 1 wk of age (Experiment 2).

Inf. profile[A]	Prebleed	2 wk	4 wk	8 wk	12 wk	16 wk	20 wk
V+A+ (%)	0	0	0	16	13	13	24
V+A− (%)	0	100	87	57	50	47	44
V−A+ (%)	0	0	6.5	23	37	40	24
V−A− (%)	100	0	6.5	3	0	0	8

[A]ALV J infection profile in commercial meat-type chickens following infection ADOL pR5-4 at 1 wk of age, V+A+ = chickens were concurrently positive for viremia (V) and neutralizing antibody response against the inoculated parental virus (NAb); V+A− = chickens are positive for V and negative for NAb; V−A+ = chickens have cleared the viremia by developing an efficient NAb; V−A− = chickens are negative for V and NAb.

RESULTS

ALV J infection profile in chickens infected with ADOL pR5-4. The results of Experiment 1 are depicted in Table 2. In commercial meat-type chickens inoculated at hatch with ADOL pR5-4 and contact-exposed meat-type sentinel chickens, the incidence of V+A+ ALV J infection profile was 88% and 23%, respectively. In comparison, it was 4% in line 0 chickens inoculated at hatch. None of the meat-type chickens inoculated at hatch was able to clear viremia, even though 88% of the chickens had NAbs against the inoculated parental virus. In contrast, 77% of contact-exposed meat-type sentinel chickens and 93% of ADOL line 0 chickens were able to produce an efficient NAb response that is able to clear viremia.

In Experiment 2 (Table 3), commercial meat-type chickens that were inoculated at 1 wk of age with ADOL pR5-4 had a V+A+ status in 13–24% of the chickens. By the time of study termination at 20 wk, the total percentage of viremic chickens is 68%, and 44% of these chickens remained viremic without producing any NAb against the inoculated parental virus (V+A−), and 24% of the viremic chickens did produce NAb against the inoculated parental virus but were unable to clear the concurrent viremia (V+A+). Also 48% of chickens produced neutralizing antibody response against the inoculated parental virus, but only 24% of these chickens cleared concurrent viremia (V−A+), and the other 24% of the chickens remained viremic (V+A+) since their NAb could not neutralize the concurrent circulating virus. All the *in ovo* inoculated chickens remained viremic with no NAb responses (data not presented).

NAb responses against autologous viruses. The samples were collected from meat-type chickens with V+A+ profile during the last four to six sampling intervals before the study was terminated at 32 wk PH in Experiment 1 and at 20 wk PI in Experiment 2. The results are presented in Table 1. Antibodies isolated at all sampling intervals were able to neutralize the inoculated parental virus (V+A+). In the selected V+A+ chickens inoculated with ALV J field isolates (ADOL Hc1, ADOL 4817, and ADOL 6803), the autologous viruses were neutralized in 0% to 50% of the antibody samples (samples 1–5). In the selected V+A+ chickens from Experiment 1 (samples 6–15) that were inoculated with ADOL pR5-4 and raised as a flock in a floor pen, the autologous viruses were neutralized in 0% to 66% of the antibody samples. However, in the selected V+A+ chickens from Experiment 2 (samples 16–18) that were inoculated with ADOL pR5-4 and raised in isolation in solitary HB units, none (0%) of the autologous viruses were neutralized.

The VN matrix pattern between autologous and heterologous NAbs of the three chickens from Experiment 2 that are inoculated with ADOL pR5-4 and raised in isolation in individual HB units is illustrated (Fig. 1A). In addition, an example of a VN matrix pattern from the chickens infected with ALV J field isolates as well as chickens infected with ADOL pR5-4 and raised as a flock in a floor pen is also illustrated (Fig. 1B). As indicated above, the majority of the V+A+ chickens were unable to neutralize autologous viruses. However, some of these resilient viruses were neutralized by antibodies (heterologous) from later sampling intervals. Some differences were found in heterologous VN of viruses, especially in

A.	A1[3]	A2[3]	A3[3]	A4[3]	A5[3]
V0[1]	+	+	+	+	+
V1[2]	-	+	+	+	+
V2[2]	-	-	+	+	+
V3[2]	-	-	-	+	+
V4[2]	-	-	-	-	-
V5[2]	-	-	-	-	-

B.	A1[3]	A2[3]	A3[3]	A4[3]	A5[3]
V0[1]	+	+	+	+	+
V1[2]	+	+	+	+	-
V2[2]	-	-	+	+	+
V3[2]	-	+	-	+	+
V4[2]	+	+	-	-	+
V5[2]	-	-	-	-	-

Fig. 1. Examples of different patterns of virus neutralization (VN) matrix. (A) Autologous VN was absent in all cases but most of the emergent viruses were neutralized by later antibodies. None of the virus isolates were neutralized by antibodies from earlier samplings. Sequential virus isolates from all three chickens from Expt. 2 and a few chickens from Expt. 1 shared similar VN matrix patterns. (B) Autologous VN was absent in all cases except V1 and A1. Most of the emergent viruses were neutralized by later antibodies. Some of the virus isolates were also neutralized by antibodies from earlier samplings. Chickens raised as a flock in a floor pen shared similar VN matrix patterns. [1]Parental virus that was used to inoculate the chickens; [2]Viruses (V1–V5) isolated at various sampling intervals (8, 16, 24, 28, and 32 wk PH in Expt. 1 or 8, 12, 16, and 20 wk PH in Expt. 2); [3]Antibodies (A1–A5) isolated at various sampling intervals (8, 16, 24, 28, and 32 wk PH in Expt. 1 or 8, 12, 16, and 20 wk PH in Expt. 2).

10055	Ab from 10075				
	A0	A8	A12	A16	A20
V0	+	+	+	+	+
V2	-	+	+	+	-
V4	-	+	+	+	-
V8	-	-	-	-	-
V12	-	-	-	-	-

10075	Ab from 10055			
	A0	A8	A12	A16
V0	+	+	+	-
V2	-	+	+	+
V4	-	+	+	+
V8	-	-	-	-
V12	-	-	-	-

Fig. 2. The VN neutralization matrix between sequential virus isolates from one chicken (10055) and antibody samples from a different chicken (10075) and *vice versa* are depicted. Both the chickens were inoculated at 1 wk of age with a molecular clone ADOL pR5-4 and raised in isolation in individual HB units. [1]Parental virus (V0) and antisera (A0) against the parental virus; [2]Viruses isolated at various time points (2, 4, 8, and 12 wk) postinoculation; [3]Antibodies isolated at various time points (8, 12, 16, and 20 wk) postinoculation. Twenty wk antibody samples were not available from chicken 10055.

chickens that are raised as a flock in a floor pen. In some cases, viruses were neutralized by earlier antibodies, while in other cases, viruses were not neutralized by antibodies from later sampling intervals (an example is illustrated in Fig. 1B). In addition, results from the cross VN matrix assay using the sequential virus isolates and antibody samples from chickens 10055 and 10075 from Experiment 2 are depicted in Fig. 2. The results indicated that the antibody samples isolated from one chicken (10055) could neutralize the virus isolates of another chicken (10075) and *vice versa*.

Sequence analysis of the *env* gene of the virus isolates from Experiment 2. PCR amplification using 6–2 primers yielded a DNA product of about 2.4 kb from the DNAs extracted from CEFs infected with ADOL pR5-4 and its variants from all the three chickens (10022, 10075, 10089) at 2, 4, 8, 12, 16, and 20 wk PI. As indicated in Fig. 3A,B, there are long stretches of conserved AA sequences interspersed with multiple hypervariable regions within the gp85 and gp37 region. The hypervariable stretches of AA within the gp85 region include 104, 116–135, 153–178, 192–221, 242–265, and 310, and within the gp37 region include 313–324, 329–337, 349, 376, 377, 404, and 421. The %AA sequence divergence between ADOL pR5-4 and its variant viruses isolated from chickens 10022, 10075, and 10089 was 5.3%, 4.1%, and 3.9%, respectively (data not presented). Cysteine residues are considered to play a vital role in generating and/or maintaining a tertiary structure of the envelope proteins that is important for various stages of the viral life cycle (21). All the 14 cysteine residue sites within the gp85 region of ADOL pR5-4 and its variant viruses were conserved, indicating maintenance of the essential tertiary structure of the ALV J envelope. There were 14 N-linked glycosylation (Asn-Xaa-Ser/Thr) sites within the gp85 region of ADOL pR5-4. The N-linked glycosylation sites within the AA sequences were well conserved except at AA 71 (in chicken 10022 at 12, 16, and 20 wk isolate), at AA 218 (in chicken 10022 at 20 wk isolate), and at AA 242 (in chicken 10075 at 12 wk isolate and in chicken 10089 at 12 and 16 wk isolates). Overall, there was a gain of 1–2 glycosylation sites within the gp85 region in isolates from 12 wk postinoculation onward. The NS/S ratios for the *env* (gp85, gp37) region for 10022, 10075, and 10089 chicken isolates at 20 wk PI were 2.45 (5.33, 1.37), 1.35 (2.11, 0.73), and 2.08 (3.7, 1.1), respectively (Table 4).

The phylogenic dendrogram (Fig. 4) indicates that the early virus isolates (2–8 wk PI) in all three chickens clustered together very close to the parental ADOL pR5-4, and divergence from ADOL pR5-4 was seen in isolates from 12 wk PI onward. Remarkably, some of the later isolates were also closely related, as indicated by the close relationship between 16 and 20 wk isolates of 10022 and 10075. This association was also confirmed by the AA sequence analysis (Fig. 3A,B). Another interesting observation was that AAs at certain positions tended to mutate and change to common AAs in multiple virus isolates. For example, AAs at position 104, 131, 161, 192, 197, 211, 244, and 310 in 16 and 20 wk isolates from chickens 10022 and 10075 mutated to common AAs lysine, phenylalanine, glutamine, proline, asparagine, serine, proline, and arginine, respectively. Even though the 20 wk PI isolate from chicken 10089 was not as closely related to the other two viruses, there were positions that also tended to mutate to common AAs. Some examples include AAs at positions 104, 161, 242, and 310 started out as glutamic acid, arginine, serine, and lysine in the parental virus ADOL pR5-4, but by 20 wk PI, isolates from all three chickens had lysine, glutamine, lysine, and arginine, respectively.

DISCUSSION

In this paper, we provide data to demonstrate the presence of NAb escape variants in ALV J infections and their contribution to high levels of viral persistence in meat-type chickens. We have previously shown that there is a high incidence of viral persistence even in the face of an efficient NAb response against the inoculated parental virus (V+A+) in meat-type chickens infected with ALV J field isolates (13). In this study, we have demonstrated a similar high incidence of viral persistence in the presence of an efficient NAb response using an ALV J molecular clone ADOL pR5-4 in commercial meat-type chickens. Also, as previously reported after infection with ALV J field isolates (12), the viral persistence in meat-type sentinel chickens as well as ADOL line 0 chickens infected with ADOL pR5-4 was very low, since these chickens were able to clear ALV J viremia by producing an efficient NAb response. The ability

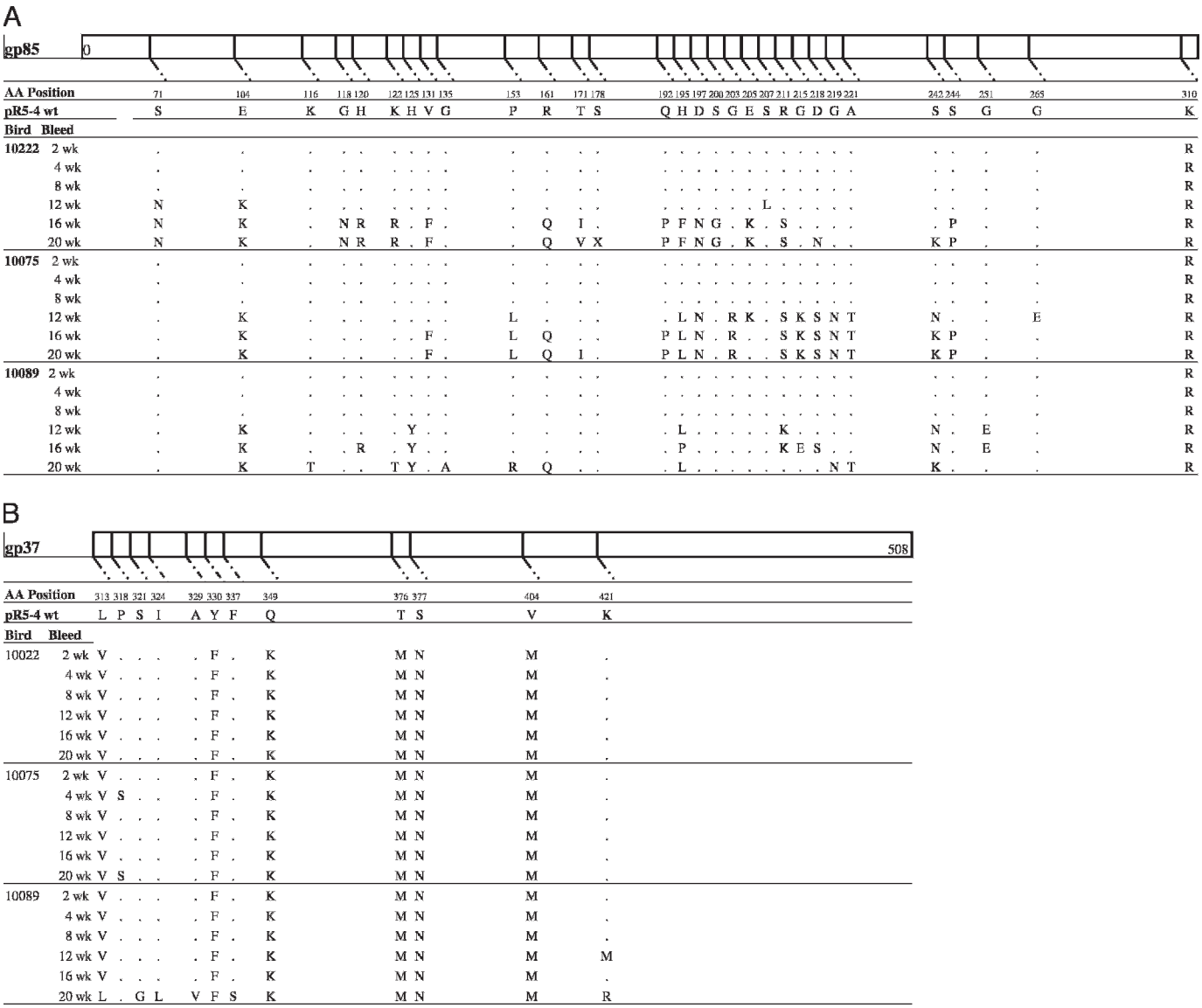

Fig. 3. The amino acid sequences within the gp85 region (3A) and gp37 region (3B) of all the variant viruses isolated from chickens 10022, 10075, and 10089 at 2, 4, 8, 12, 16, and 20 wk PI are compared to the AA sequence of the parental virus ADOL pR5-4. The position of the AA that have mutated within the variant viruses are indicated in the row labeled ''AA position,'' and the original AA within the inoculated ALV J ADOL pR5-4 is indicated in the row labeled ''pR5-4 wt.'' E = glutamic acid; F = phenylalanine; K = lysine; N = asparagine; P = proline; Q = glutamine; R = arginine; S = serine.

of meat-type sentinel chickens to develop a neutralizing antibody response and clear the viremia may be related to the later age of infection and possibly lower titer of challenge exposure from contact with infected chickens. The ability of ADOL line 0 chickens to develop a neutralizing antibody response and clear the viremia may be related to host genetics and lack of endogenous *ev* loci.

The presence of viremia in the face of an efficient NAb response against the inoculated parental virus indicated that the virus may have mutated to escape immune pressure elicited by the NAb. The relevance of NAb escape variants in viral persistence has been demonstrated in other viruses, such as EIAV (6), visna virus (10), LCMV (1), HIV (24,16), and FMDV (8).

Analyses of the VN matrix assays provided relevant information regarding autologous and heterologous NAb. The complexity of the NAb response was demonstrated since the VN matrices did not have the same profile even though all chickens were V+A+ at every sampling interval (compare Fig. 1A to Fig. 1B). In chickens infected with the molecular clone ADOL pR5-4 and raised in isolation in individual HB units, heterologous VN was observed only in virus isolates collected from earlier time periods but not later time periods. However, in chickens inoculated with the field viruses or ADOL pR5-4 and raised as a flock in a floor pen, the heterologous VN was observed in some virus isolates collected from earlier as well as later time periods. This discrepancy has several possible causes: the field strains usually consist of a heterogeneous mixture of viruses; the chickens in Experiment 1 were reared in a flock, and mixtures of viruses derived from the ALV J molecular clone ADOL pR5-4 in various chickens can be circulating in the flock; and chickens can produce antibodies against those mixtures of viruses. The latter point is validated by the fact that in Experiment 2, antibodies isolated from one chicken (10055) were able to neutralize virus isolates from a different chicken (10075) and *vice versa* (Fig. 2).

Routine testing in ALV J infection experiments involves virus isolation from plasma as well as testing for NAb against the

Table 4. The number of synonymous and nonsynonymous amino acid substitutions within the gp85 (SU) and gp37 (TM) region as well as the NS/S ratios from the 20 wk isolates of chickens 10022, 10075, and 10085.

Variant	Region	No. of amino acid changes[A] Synonymous (S)	Nonsynonymous (NS)	NS/S ratio
10022-20	Env	11	27	2.45
	gp85 (SU)	3	16	5.33
	gp37 (TM)	8	11	1.37
10075-20	Env	20	27	1.35
	gp85 (SU)	9	19	2.11
	gp37 (TM)	11	8	0.73
10089-20	Env	13	27	2.08
	gp85 (SU)	4	15	3.75
	gp37 (TM)	11	12	1.09

[A]The relative rates of AA change was measured by calculating the ratio of nonsynonymous (NS, AA changing) to synonymous (S, AA preserving) AA substitution patterns based on the methods described by Nei and Gojobori (11).

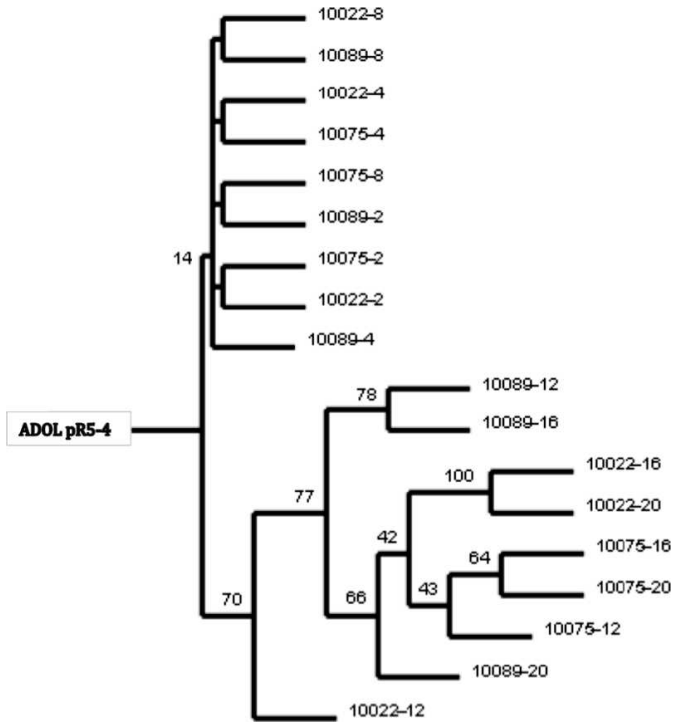

Fig. 4. Phylogenic dendrogram illustrating the relationship between the *env* (gp85 and gp37) gene sequences of the parent strain ADOL pR5-4 and its variants isolated from chickens 10022, 10075, and 10089 at 2, 4, 8, 12, 16, and 20 wk postinoculation. The amino acid sequences were aligned by using ClustalX method, and the dendrogram was generated by using PHYLIP, version 3.6, maximum likelihood method for amino acid sequences. In this bootstrap analysis, the numbers on the branches indicate the number of times the viruses on the right descended from the fork in 100 samples. The isolates from 2, 4, and 8 wk postinoculation in all the three chickens cluster together, and the divergence increases from 12 wk onward.

inoculated (parental) virus at different sampling intervals. This work has demonstrated that routine description of ALV J antibody (A+ or A−) is not entirely accurate since the NAb response is always against the inoculated virus but not the autologous virus. As discussed earlier, this does give some information about the immune response of the chicken, but it misses important information on the emergence of variant viruses. On the other hand, testing for sequential autologous antibody response is cumbersome, since this involves virus isolation from plasma obtained from several sampling intervals, preparation of viral stocks, and biological titration in tissue culture by limiting dilution.

Variables like heterogeneity of viral inoculum and spread of viral variants in a flock were avoided in Experiment 2, where chickens were inoculated with an ALV J molecular clone ADOL pR5-4 and reared in isolation in individual HB units. Sequencing of virus isolates collected at different time intervals from these chickens with concurrent V+A+ status provided important information about the molecular changes within the *env* gene as a result of the highly selective immune pressure elicited by the neutralizing antibodies. The antibody escape viral variants as measured by the VN matrix assay appeared between 8 and 12 wk PI. Nucleotide sequence and the deduced AA sequence analysis indicated regions of hypervariability interspersed with stretches of conserved regions with the gp85 region showing a higher degree of sequence variability than the gp37 region. The hypervariable regions 116–135 and 192–221 within the gp85 region and 313–337 within the gp37 region match with the hypervariable regions described by Silva *et al.* (19), as well as with the hr1–hr2 and gp37 regions described by Venugopal *et al.* (22). As expected in this study, the %AA divergence of variants derived from the ALV J molecular clone ADOL pR5-4 was lower compared to the %AA divergence in the field isolates described by Silva *et al.* (19) and Venugopal *et al.* (22). Examination of the AA NS/S ratios indicates the molecular evolution to escape selection pressure such as the host immune responses (9,18). The NS/S ratios were higher within the surface (SU) (gp85) protein region than the transmembrane (TM) (gp37) protein region as observed within the lentiviruses (9). The higher NS/S ratios within the gp85 than the gp37 region indicate that the gp85 region is mutated to a higher degree due to immune pressure. The gp85 NS/S ratios described in this study (Table 4) were comparable to the data by Wang and Cui (23), where they passaged ALV J in CEFs supplemented with subneutralizing doses of antisera against ALV J. However, the gp85 NS/S ratios described in this study are higher than the ratios described by Venugopal *et al.* (22) in field isolates (1.77), where only 2/12 ALV J isolates were neutralized by antisera against Houghton Poultry Research Station (HPRS)-103, the English prototype of ALV J (22). These findings are remarkable, especially since the increase in the NS/S ratios of the gp85 region in the experiments of Wang and Cui (23) was solely due to the selection pressure of the neutralizing antibodies, thereby supporting the premise that the variants in our study were the product of selection pressure induced by neutralizing antibodies.

Based on the phylogenic analysis, the earlier isolates (2, 4, and 8 wk PI) clustered together very close to the parental virus ADOL pR5-4 as expected, since the AA sequence variations appeared between 8 and 12 wk PI. One noteworthy feature was that some of the latter (16 and 20 wk PI) virus isolates also clustered together, even though they were derived from different chickens and after several different mutations. Remarkably, the AA sequence analysis of these virus isolates (derived from different chickens at various time points) indicated that AA at certain positions eventually mutate to the same/common AA. This is exemplified by the mutation of AAs at positions 104, 131, 161, 192, 197, 211, 244, and 310 in 16 and 20 wk isolates from chickens 10022 and 10075 to common AAs lysine, phenylalanine, glutamine, proline, asparagine, serine, proline, and arginine, respectively. Even though the 20 wk PI isolate from chicken 10089 was not as closely related to the other two viruses, there were positions that also tend to mutate to common AAs. These

results indicated that the above AA substitutions were not a series of random nucleotide mutations that resulted in AA changes, but rather were the product of a highly selective immune pressure to escape from the prevailing NAb responses. It is therefore very tempting to speculate that these AA (regions) may contain neutralizing epitopes of the ALV J envelope protein, especially since all these viral variants are not neutralized by antisera against the parental virus. Further studies aimed at site directed mutagenesis of these AAs and developing neutralizing monoclonal antibodies against ALV J may validate the claim that these indeed comprise at least some neutralizing epitopes in ALV J.

The high genetic variation in retroviruses is due the low replication fidelity of reverse transcriptase that lacks 3′ to 5′ exonuclease ''proofreading'' activity, which leads to a highly error prone replication at a rate of 10^{-4} per nucleotide. The virus replicates to high titers and forms variably differentiated/related pools of viral populations that exist as quasispecies. Also, in the face of selective immune pressure, these viral populations mutate to overcome the host immune responses and form novel stable variants that persist in the susceptible host populations. In this study, the virus isolates were derived from a homogenous molecular clone ADOL pR5-4 and were not neutralized by neutralizing antibodies against the parental virus. In addition, some of the viruses were also not neutralized by autologous viruses, indicating that these were indeed antibody escape variants. The inability of the host to produce neutralizing antibodies against the escape variants even after 8 wk of emergence may be due to fact that these variants may be less immunogenic as a result of developing novel N-linked glycosylation sites that mask the immunogenic polypeptide epitopes or cause confirmational changes within the epitopes making them less immunogenic. These antigenic variants can persist in the host and can potentially be transmitted vertically. Vertical transmission of neutralization escape variants of human immunodeficiency virus (HIV)-1 has been demonstrated in humans (27). It would of considerable interest from the ALV J epidemiology perspective to compare virus isolates from the dams as well as from the chicks infected by vertical transmission.

In this study, we have demonstrated the presence of ALV J NAb escape variants and their role in the high incidence of V+A+ infection profile and viral persistence. These results are very significant in the context of epidemiology of ALV J in meat-type chickens. This is also very important in view of reports of ALV J infection in egg-type chickens, indicating an increase of host range (28). The demonstration of antibody escape variants in just a few birds within a span of 20 wk indicates a high potential of this virus to mutate under field conditions in large flocks. The high level of viral persistence observed in meat-type chickens is of concern for the ALV J eradication programs as a result of the increased potential for viral evolution.

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